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# Purification and characterization of an intracellular cycloalternan-degrading enzyme from *Bacillus* sp. NRRL B-21195

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**Abstract**—A novel intracellular cycloalternan-degrading enzyme (CADE) was purified to homogeneity from the cell pellet of *Bacillus* sp. NRRL B-21195. The enzyme has a molecular mass of 125 kDa on SDS-PAGE. The pH optimum was 7.0, and the enzyme was stable from pH 6.0 to 9.2. The temperature optimum was 35 °C and the enzyme exhibited stability up to 50 °C. The enzyme hydrolyzed cycloalternan [CA; *cyclo*{→6)-α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-α-D-Glcp-(→3)-α-D-Glcp-(1→)}] as the best substrate, to produce only isomaltose via an intermediate, α-isomaltosyl-(1→3)-isomaltose. This enzyme also hydrolyzed isomaltosyl substrates, such as panose, α-isomaltosyl-(1→4)-maltooligosaccharides, α-isomaltosyl-(1→3)-glucose, and α-isomaltosyl-(1→3)-isomaltose to liberate isomaltose. Neither maltooligosaccharides nor isomaltoligosaccharides were hydrolyzed by the enzyme, indicating that CADE requires α-isomaltosyl residues connected with (1→4)- or (1→3)-linkages. The  $K_m$  value of cycloalternan (1.68 mM) was 20% of that of panose (8.23 mM). The  $k_{cat}$  value on panose (14.4 s<sup>-1</sup>) was not significantly different from that of cycloalternan (10.8 s<sup>-1</sup>). Judging from its specificity, the systematic name of the enzyme should be cycloalternan isomaltosylhydrolase. This intracellular enzyme is apparently involved in the metabolism of starch via cycloalternan in *Bacillus* sp. NRRL B-21195, its role being to hydrolyze cycloalternan inside the cells.

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**Keywords:** Cycloalternan; Cycloalternan-degrading enzyme, CADE; Cycloalternan isomaltosylhydrolase; *Bacillus* sp. NRRL B-21195

## 1. Introduction

Cycloalternan (CA) is a cyclic tetrasaccharide [*cyclo*{→6)-α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-α-D-Glcp-(→3)-α-D-Glcp-(1→)}] with alternating α-(1→3)- and α-(1→6)-linkages.<sup>1</sup> In crystalline form it shows a plate-like shape with a shallow depression in the center. Its structure is not fully symmetrical because of an asymmetric hydrogen bond.<sup>2</sup> It has been proposed that this substrate may be useful as a functional additive in food.<sup>3</sup> Côté and Biely<sup>4</sup> reported that cycloalternan is produced from alternan (alternating α-(1→3), α-(1→6)-D-glucan) by the action of alternanase from *Bacillus* sp. NRRL

**Abbreviations:** CA, cycloalternan; CAFE, cycloalternan-forming enzyme; CADE, cycloalternan-degrading enzyme; CD, cyclomaltodextrin; CDase, cyclodextrinase; CGTase, cyclodextrin glucanotransferase; DE, disproportionating enzyme; d.p., degree of polymerization; HPIC, high-performance ion chromatography; IM(1→3)G, α-isomaltosyl-(1→3)-glucose; IM(1→3)IMG<sub>n</sub>, α-isomaltosyl-(1→3)-α-isomaltosyl-(1→4)-maltooligosaccharide; IMG<sub>n</sub>, α-isomaltosyl-(1→4)-maltooligosaccharide; MOPS, 3-[N-morpholino]propanesulfonic acid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis  
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B-21195, possibly via some intermediates. However, the yield of cycloalternan from alternan was not more than 30%, showing that this reaction is not effective for the quantitative production of cycloalternan.

Later, it was reported that two extracellular enzymes of *B. globisporus* C11 synergistically produced cycloalternan from maltodextrin (maltooligosaccharide mixture) or starch.<sup>5–7</sup> Recently we also found independently that the role of the alternanase is to produce cycloalternan from maltodextrin or from starch by acting in concert with another enzyme (disproportionating enzyme, DE).<sup>8</sup> Therefore, alternanase was renamed cycloalternan-forming enzyme (CAFE).<sup>8</sup> The synergistic reaction mechanism for the production of cycloalternan from maltodextrin or starch is proposed to be as follows: Firstly, DE transfers an  $\alpha$ -D-glucopyranosyl unit from the nonreducing end of one maltooligosaccharide or starch chain to that of another, forming an  $\alpha$ -(1 $\rightarrow$ 6)-linkage. This results in an isomaltosyl maltooligosaccharide (IMG<sub>n</sub>). In the next step, CAFE reacts with the IMG<sub>n</sub> by transferring the isomaltosyl residue to the nonreducing end of another IMG<sub>n</sub> to produce  $\alpha$ -isomaltosyl-(1 $\rightarrow$ 3)- $\alpha$ -isomaltosyl-(1 $\rightarrow$ 4)-maltooligosaccharides [IM(1 $\rightarrow$ 3)IMG<sub>n</sub>]. Then CAFE catalyzes intramolecular transglycosylation, or cyclization, of IM(1 $\rightarrow$ 3)IMG<sub>n</sub> to produce cycloalternan. By the synergistic reaction of CAFE and DE, cycloalternan can be produced from maltodextrin or starch in high yield. These two enzymes (CAFE and DE) are produced extracellularly from *Bacillus* sp. NRRL B-21195 and can be purified with ease using isomaltose affinity chromatography.<sup>9</sup> The enzyme systems of *Bacillus* sp. NRRL 21195<sup>8</sup> and *B. globisporus*<sup>5</sup> seem identical based on their action, but the N-terminal sequences of the two enzymes are different.

A number of cyclic oligosaccharides have been produced by enzymatic methods.<sup>10–12</sup> Among them, cyclomaltodextrins (CDs) are the most widely known and best studied. The cyclomaltodextrins are formed enzymatically from starch by the action of cyclomaltodextrin glucanotransferase (CGTase).<sup>13–15</sup> The cyclomaltodextrins are not normally hydrolyzed by *exo*-type enzymes, but are hydrolyzed by *endo*-type enzymes, such as maltogenic  $\alpha$ -amylases.<sup>16,17</sup> Recently, Hashimoto et al.<sup>18</sup> reported that *Thermococcus* sp. strain B1001 possesses five proteins related to cyclomaltodextrins, namely, cyclomaltodextrin glucanotransferase (CGTase), cyclomaltodextrinase (CDase), CD-binding protein (CBP), and two components of CBP-dependent ATP-binding cassette (ABC) transporter. Extracellular CGTase produces CD from starch, and the resultant CDs are transported into the cells by the action of a transporter. Finally, the intracellular CDase hydrolyzes the cyclomaltodextrins. Fiedler et al.<sup>19</sup> also reported a similar starch utilization pathway via CDs to be present in *Klebsiella oxytoca*. They

showed that the CDase is intracellularly expressed and involved in the hydrolysis of CDs.

Currently, there are three bacterial strains, *Bacillus* sp. NRRL B-21195,<sup>1,4</sup> *B. globisporus* C11,<sup>5,6</sup> and *Arthrobacter* sp.<sup>20</sup> known to produce cycloalternan from maltodextrin and starch. Though a gene cluster encoding five CA-related enzyme genes (ctsU, V, W, Y, and Z) have been cloned and sequenced from *B. globisporus* C11, no CA-degrading enzyme has yet been described from that strain.

Based on the metabolic pathways of CDs present in *Thermococcus* sp. strain B1001<sup>18</sup> and *K. oxytoca*,<sup>19</sup> we supposed that there would be an enzyme involved in the hydrolysis of cycloalternan in *Bacillus* sp. NRRL B-21195. To discover such an enzyme, we examined both intracellular and extracellular CA-degrading activity. As a result, we found a novel intracellular CA-degrading activity showing much higher activity on cycloalternan than on any other substrates tested. The novel enzyme cycloalternan-degrading enzyme (CADE) was subsequently purified and characterized. A potential role of this enzyme in the catabolism of starch via cycloalternan is discussed. To the best of our knowledge, this is the first enzyme that hydrolyzes cycloalternan specifically.

## 2. Materials and methods

### 2.1. Materials

Maltooligosaccharides (G<sub>2</sub>–G<sub>7</sub>) were purchased from Kokusan Laboratory Chemicals (Tokyo, Japan) and Hyashibara Biochemical Laboratories (Okayama, Japan). Cycloalternan was prepared by the action of CAFE from alternan.<sup>4</sup> Panose was purchased from Hyashibara Biochemical Laboratories.  $\alpha$ -Isomaltosyl-(1 $\rightarrow$ 3)-D-glucose [IM(1 $\rightarrow$ 3)G] was prepared by the action of *Leuconostoc mesenteroides* NRRL B-512F dextranucrase acceptor reaction<sup>21</sup> with nigerose.  $\alpha$ -Isomaltosyl-(1 $\rightarrow$ 4)-maltooligosaccharides (IMG<sub>n</sub>) were prepared by the action of *Bacillus* sp. NRRL B-21195 DE.<sup>8</sup>  $\alpha$ -Isomaltosyl-(1 $\rightarrow$ 4)-maltose (IMG<sub>2</sub>) was also synthesized by using isomaltodextranase from *Arthrobacter globiformis* T6.<sup>22</sup>  $\alpha$ -Isomaltosyl-(1 $\rightarrow$ 3)-isomaltose [IM(1 $\rightarrow$ 3)IM] was prepared from a partial hydrolyzate of cycloalternan using the action of CADE. All other chemicals were of reagent grade and used without further purification.

### 2.2. Bacterial growth and production of enzyme

*Bacillus* sp. NRRL B-21195 obtained from the National Center for Agricultural Utilization Research (Peoria, IL) was grown at 30 °C on a rotary shaker in the medium described by Ahlgren and Côté.<sup>9</sup> For production of

larger amount of CADE, 5 L of Erlenmeyer-flask-grown cultures, each containing 1 L of the standard tryptone–soy medium with glucose were inoculated from starter cultures and grown with shaking at 30 °C for 3 days. Cells were harvested by centrifugation (17,000g for 30 min at 4 °C), washed twice with 50 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS) buffer (pH 7.0), and sonicated (Branson sonifier 250/450 D) in the same buffer. The cell debris was removed by centrifugation (15,000g for 10 min at 4 °C) and the resultant supernatant was used as the crude enzyme preparation.

### 2.3. Purification of CADE

The crude enzyme preparation was applied to a DEAE Toyopearl 650S column (5 mL, Tosoh, Tokyo, Japan) equilibrated with 50 mM MOPS buffer (pH 7.0) and eluted with a 0–0.5 M NaCl gradient. The fractions showing hydrolytic activity on cycloalternan and panose were collected and reloaded onto a 1 mL HiTrapQ (Amersham Biosciences, Piscataway, NJ) column and eluted with a 0–0.3 M NaCl gradient in 50 mM MOPS buffer. To remove minor contaminants, the active fractions were collected and loaded onto a 1 mL MonoQ column (Amersham Biosciences), and eluted with a 0–0.3 M NaCl gradient in 50 mM MOPS buffer (pH 7.0). Protein concentration at each step was estimated by absorbance at 280 nm using bovine serum albumin (BSA) as the standard.

### 2.4. Analytical methods

SDS-PAGE was performed by the method of Laemmli<sup>23</sup> using 9% acrylamide containing 0.1% SDS. The gel was stained in 0.1% Coomassie Brilliant BlueR-250–50% MeOH–10% AcOH. For the purpose of amino acid sequencing, the enzyme separated on SDS-PAGE was transferred onto poly(vinylidene difluoride) membrane by electro-blotting (BioRad electroblotting system, Hercules, CA, USA). N-Terminal sequences of the blotted samples were determined using a protein sequencing system (Model G1001A; Hewlett-Packard Co., Corvallis, OR, USA). To determine the native molecular mass of the enzyme, the protein was loaded onto a gel filtration column (Superdex 200 HR10/30; Amersham Biosciences) pre-equilibrated with 0.15 M NaCl in 50 mM sodium phosphate buffer, pH 7.0. The column contents were eluted at flow rate of 0.5 mL/min. MW-Marker HPLC (Oriental Yeast Co., Tokyo, Japan) was used as a molecular-mass standard.

The reaction products of the CADE were analyzed by high-performance ion chromatography (HPIC, Dionex DX320, Dionex, Sunnyvale, CA) using a Dionex Carbpac PA1 column (4×250 mm) as described by

Percy et al.<sup>24</sup> The column was eluted with a linear gradient of NaOAc (0–200 mM) in 100 mM NaOH at a flow rate of 1 mL/min. The elution gradient was performed over a period of 20 min.

### 2.5. Effect of pH and temperature

To determine the optimum pH of the CADE, enzyme activity was determined at 35 °C using the following buffers at 50 mM concentration: NaOAc acetate (pH 3.8–6.0), MOPS (pH 5.8–7.8), *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) (pH 7.8–8.6), and cyclohexylaminopropanesulfonic acid (CHAPS) (pH 8.2–11.0). Similarly, the pH stability was determined by preincubating the enzyme at 35 °C for 30 min with the same buffers already described, and remaining activity was determined using the standard assay procedure. The temperature optimum was determined using the standard assay at pH 7.0 (50 mM MOPS) at temperatures ranging from 0 to 70 °C. For determinations of thermal stability, the enzyme was incubated for 30 min at different temperatures between 0 and 70 °C. After cooling the sample on ice for 10 min, the remaining activity was determined using the standard assay procedure.

### 2.6. Enzyme activity and kinetic parameters

Enzyme activity of CADE was determined by measuring the amount of reducing sugar liberated from 5 mM cycloalternan or the amount of D-glucose from 5 mM panose in 50 mM MOPS buffer (pH 7.0). The reducing value of products liberated from cycloalternan was determined by using copper bicinchoninate reagent,<sup>25</sup> whereas the amount of D-glucose liberated from panose was determined by the glucose oxidase–peroxidase method<sup>25,26</sup> with mutarotase using Glucose CII test (Wako Pure Chemical, Osaka, Japan). One unit (IU) of the enzyme activity was defined as the quantity of enzyme liberating an amount of reducing sugar equivalent to 1.0 μmol of maltose from 5 mM cycloalternan per minute at 35 °C.

To determine the apparent kinetic parameters of the hydrolytic reaction of CADE, all of the enzyme reactions were carried out under the respective standard conditions unless otherwise specified. The mixture containing substrates and enzyme ( $2.1 \times 10^{-3}$  mg protein/mL), with a total volume of 500 μL, was incubated at 37 °C. Samples were taken at intervals and treated at 80 °C for 10 min to inactivate the enzyme. The initial velocities were calculated from the linear relationship of the product concentration against reaction time (0–30 min). The values for each parameter were calculated by nonlinear regression analysis using a computer program, GraFit (Ver. 4.0, Erithacus Software, Middlesex, UK).

## 2.7. Reaction of CADE with the several substrates

To investigate the reaction patterns of CADE, the reaction solution contained 0.1 IU/mL of purified CADE and 5 mM each substrates in 50 mM MOPS buffer (pH 7.0). After reacting for proper time, 2  $\mu$ L samples were taken and analyzed by HPIC.

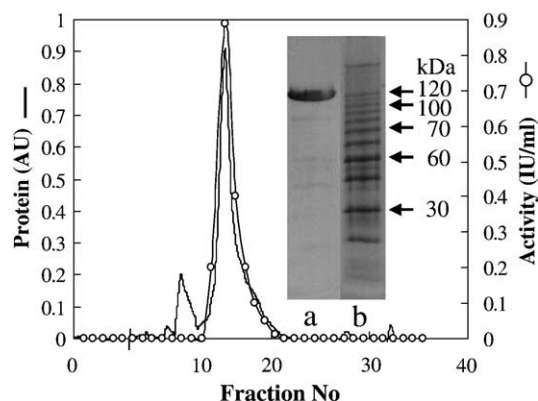
## 3. Results

### 3.1. Purification of CADE

Cycloalternan-degrading activity was detected in the cell-free extract, but not found in the culture supernatant. The purification procedure of CADE extracted from the cells is summarized in Table 1. As shown by SDS-PAGE (Fig. 1), the purified enzyme after MonoQ chromatography showed a single peak with the molecular mass of 125 kDa. A gel-permeation chromatogram of the purified enzyme also indicated a molecular mass of 120 kDa, showing the enzyme to be a monomer in aqueous condition. The N-terminal sequence of the enzyme began with serine (SYEQSTTHIGQQMLMG-YID) and no homologous sequence was found in any published protein data bank. After the purification steps, CADE was obtained in 30% yield and the specific activity on cycloalternan was 2.22 IU/mg protein.

**Table 1.** Summary of purification

Steps	Volume (mL)	Protein (mg)	Activity (IU)	Specific activity (IU/mg)	Yield (%)
Sonicate	10	365	2.92	0.01	100
DEAE-Toyo-pearl	7	8.2	1.77	0.22	61
Hi-TrapQ	3	0.8	1.38	1.75	47
MonoQ	1	0.4	0.91	2.22	30



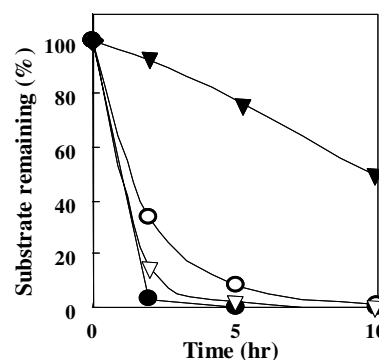
**Figure 1.** Elution profiles of the enzyme showing cycloalternan-degrading activity on MonoQ chromatography, and SDS-PAGE of the purified enzyme: (a) cycloalternan-degrading activity, (b) purified enzyme, (c) 10 kDa protein marker.

### 3.2. Effects of pH and temperature on the enzyme activity

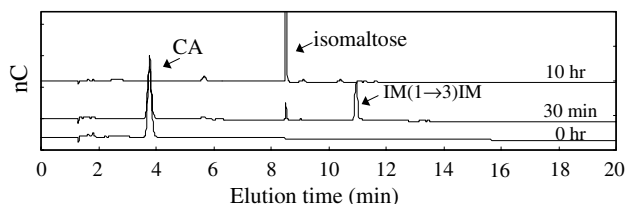
The pH optimum and stability of CADE were at pH 7.0 and pH 6.0–9.2, respectively. When the enzyme was treated at pH 5.0 and pH 9.7 for 1 h at 4 °C, the activity was decreased by 70 and 37%, respectively, relative to that at pH 7.0. The temperature optimum and stability were 35 °C and up to 50 °C, respectively. After 30 min at 55 °C, the activity was decreased to 20% of that of the untreated enzyme. The enzyme was completely inactivated after 30 min at 70 °C.

### 3.3. Reaction of CADE

CADE hydrolyzed cycloalternan into two molecules of isomaltose. It also hydrolyzed panose and IM(1  $\rightarrow$  3)G into isomaltose and glucose, and IM(1  $\rightarrow$  3)IM (the open-chain form of cycloalternan) into two molecules of isomaltose. It did not hydrolyze maltooligosaccharides, isomaltooligosaccharides, starch, dextran, pullulan, or alternan. Judging from the specificity, CADE hydrolyzes  $\alpha$ -(1  $\rightarrow$  3) or (1  $\rightarrow$  4) linked isomaltosyl residues of oligosaccharides. As shown in Figure 2, the enzyme hydrolyzed cycloalternan as the best substrate, whereas it hydrolyzed IM(1  $\rightarrow$  3)G only poorly. The enzyme was less active on the other substrates, panose and IM(1  $\rightarrow$  3)IM, relative to its activity on cycloalternan. Under identical conditions, approximately 95% of cycloalternan was degraded after 2 h reaction, whereas 85% of IM(1  $\rightarrow$  3)IM was hydrolyzed in the same period (Fig. 2). During the hydrolysis of cycloalternan, IM(1  $\rightarrow$  3)IM was initially accumulated, and ultimately hydrolyzed into isomaltose (Fig. 3). This supports our observation that CADE hydrolyzes cycloalternan faster than IM(1  $\rightarrow$  3)IM. During the hydrolysis of panose, intermediates such as IM(1  $\rightarrow$  3)IMG<sub>1</sub> or cycloalternan were not detected. These saccharides are the products from the action of CADE on panose.<sup>27</sup> Comparing the reactions on panose and IM(1  $\rightarrow$  3)G, it is obvious that



**Figure 2.** Time course of the hydrolysis of various isomaltosyl compounds: (●) cycloalternan, (○) panose, (▼) IM(1  $\rightarrow$  3)G, (▽) IM(1  $\rightarrow$  3)IM. Experimental conditions: see text.



**Figure 3.** High-performance ion chromatography of the reaction products of cycloalternan. Enzyme reaction was performed for 30 min and 10 h under standard reaction conditions, and then 2  $\mu$ L of the reaction mixture was taken for analysis of the products. CA, cycloalternan; IM, isomaltose; IM(1 $\rightarrow$ 3)IM,  $\alpha$ -isomaltosyl-(1 $\rightarrow$ 3)-isomaltose.

CADE prefers  $\alpha$ -(1 $\rightarrow$ 4) linkages over  $\alpha$ -(1 $\rightarrow$ 3) in otherwise identical trisaccharides. However, CADE hydrolyzed the tetrasaccharide IM(1 $\rightarrow$ 3)IM faster than panose, suggesting that the enzyme may recognize and bind two adjacent isomaltosyl units at its active site.

For further characterization, a series of IMG<sub>n</sub>, which were prepared by the action of DE, were examined. As shown in Figure 4, the rate at which CADE liberated isomaltose from IMG<sub>n</sub> was inversely proportional to degree of polymerization (d.p.), indicating that this enzyme prefers smaller substrates. Such a relationship of activity to d.p. was not observed with CAFE, which produces cycloalternan from IMG<sub>n</sub>.<sup>8</sup>

When the kinetic parameters for the purified enzyme were determined with panose and cycloalternan as substrates, the  $K_m$  value of cycloalternan (1.68 mM) was 20% of that of panose (8.23 mM). The  $k_{cat}$  values of panose and cycloalternan were 14.4 and 10.8 s<sup>-1</sup>, respectively, showing that the enzyme activities on both substrates were not significantly different. However the  $k_{cat}/K_m$  values on panose and cycloalternan were 1.76 and 6.44 mM<sup>-1</sup> s<sup>-1</sup>, respectively, indicating that the substrate specificity on cycloalternan was higher than

**Table 2.** Kinetic parameters of CADE

	Panose	CA
$K_m$ (mM)	8.23 $\pm$ 0.42	1.68 $\pm$ 0.14
$k_{cat}$ (s <sup>-1</sup> )	14.4 $\pm$ 0.9	10.8 $\pm$ 1.1
$k_{cat}/K_m$ (mM <sup>-1</sup> s <sup>-1</sup> )	1.76 $\pm$ 0.22	6.44 $\pm$ 0.69

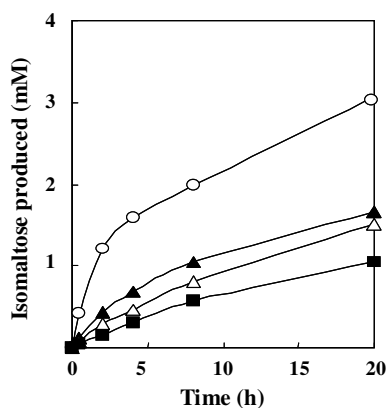
that of panose. These results strongly indicate that CADE is a new type of enzyme specific for the hydrolysis of cycloalternan (Table 2).

#### 4. Discussion

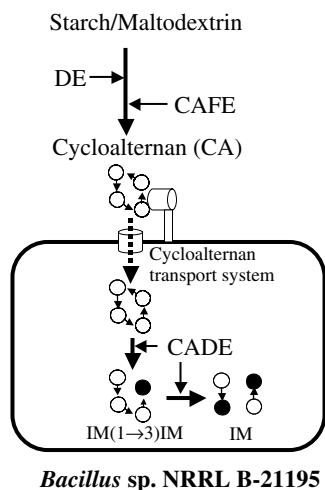
The enzyme CADE hydrolyzed various  $\alpha$ -(1 $\rightarrow$ 3)- or  $\alpha$ -(1 $\rightarrow$ 4)-linked isomaltosyl substrates. This specificity seems similar to that of CAFE, which produces cycloalternan from these substrates by an isomaltosyl-transferring reaction. The major difference between CADE and CAFE is that CADE is an isomaltosyl-hydrolase whereas CAFE is an isomaltosyl-transferase. Cycloalternan is the best substrate for CADE, suggesting that the role of CADE is to hydrolyze cycloalternan inside the cells. Judging from the specificity, the systematic name of CADE should be cycloalternan isomaltosyl-hydrolase.

Except for CADE, only two enzymes capable of hydrolyzing cycloalternan are known. One is CAFE, which possesses a slow hydrolyzing activity on cycloalternan;<sup>4,27</sup> the rate is negligible under normal reaction conditions. The other is isomaltodextranase from *A. globiformis* T6.<sup>4,28,29</sup> The major activity of the latter enzyme is an *exo*-isomaltosyl hydrolase to hydrolyze isomaltose unit from the nonreducing ends of dextran. This enzyme also retains very low *endo*-isomaltosyl hydrolase activity<sup>28,30–32</sup> (1/1000 times lower than dextranase activity) to produce isopanose from pullulan. The CA-hydrolyzing activity detected in dextran isomaltodextranase<sup>4,28,29</sup> is considered to be a result of the low level of *endo*-isomaltosyl hydrolase activity present in isomaltodextranase. The main activity of both enzymes, CAFE and isomaltodextranase, is not in the hydrolysis of cycloalternan, indicating that the 'native' role of these enzymes is not related to cycloalternan catabolism. Thus, CADE is the first enzyme found to be involved in the catabolism of cycloalternan.

Fiedler et al.<sup>19</sup> have reported a starch utilization pathway present in *K. oxytoca*. In this pathway, CGTase converts starch into cyclomaltodextrins extracellularly, then CymE transports cyclomaltodextrins into the cells and CymH hydrolyzes them into maltose and maltotriose as the end products. It indicates that this CD-producing strain possesses CDs-hydrolyzing enzyme inside the cells. Hashimoto et al.<sup>18</sup> also reported the extracellular synthesis, specific recognition, and intracellular degradation of CDs by *Thermococcus* sp. strain



**Figure 4.** Time course of the hydrolysis of IMG<sub>n</sub>. Amount of isomaltose produced from 5 mM substrates were plotted. Experimental conditions, see text: (○) IMG<sub>1</sub> (panose); (▲) IMG<sub>2</sub>; (△) IMG<sub>3</sub>; (■) IMG<sub>4</sub>.



**Figure 5.** A proposed model for the extracellular synthesis, transmembrane uptake and intracellular degradation of cycloalternan (CA) performed by *Bacillus* sp. NRRL B-21195. CAFE, cycloalternan-forming enzyme; DE, disproportionating enzyme; CADE, cycloalternan-degrading enzyme; IM(1→3)IM, isomaltosyl- $\alpha$ -(1→3)-isomaltose; IM, isomaltose.

B1001. They proposed the existence of a cyclomaltodextrin transport system and CDase inside the cells.

The role of intracellular CADE seems to resemble that of intracellular CDase. To explain the extracellular synthesis<sup>8</sup> and intracellular hydrolysis of cycloalternan, a model of the catabolic pathway of starch via cycloalternan is proposed (Fig. 5). As shown in Figure 5, the synergistic reaction of CAFE and DE first produces cycloalternan from starch outside the cells. Then, the resultant cycloalternan is transported into the cells by a cycloalternan transport system. The transported cycloalternan is finally degraded into isomaltose intracellularly by the action of CADE. Genes of a possible transporting system for cycloalternan have been found upstream of the cycloalternan forming enzymes in *B. globisporus*.<sup>5,6</sup> Studies on the cycloalternan transport system in *Bacillus* sp. NRRL B-21195 are currently in progress.

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